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EFFECT OF CALINA PAPAYA LEAVES EXTRACT ON RESPIRATORY TRACT IN CIGARETTE SMOKE EXPOSED RATS

Haris Setiawan[1*], Arlinda Sekar Ayu Hikmawati[2], Sri Wijayanti Wulandari[1], Eriza Via Malinda[1], Ichsan Luqmana Indra Putra[1]

[1,3,4,5]Departement of Biology, Faculty of Applied Science and Technology, Universitas Ahmad Dahlan, Daerah Istimewa Yogyakarta 55166
[2]Departement of Biomedical Science, Faculty of Medicine Publich Health and Nursing, Universitas Gadjah Mada, Daerah Istimewa Yogyakarta, 55281

Email : [1*]haris.setiawan@bio.uad.ac.id, [2]arlindasekar12@gmail.com, [3]sri.wijayanti@bio.uad.ac.id, [4]erizaviamalinda04@gmail.com, [5]ichsan.luqmana@bio.uad.ac.id

Abstract. *Cigarette smoke is a source of free radicals that cause health problems throughout the world. Indonesia is a country that has many sources of natural antioxidants in counteracting free radicals, one of which is Calina papaya leaves. The study aimed to determine the potential of Calina papaya leaves antioxidants in improving the respiratory organs of Wistar rats after being exposed to cigarette smoke. The study using 25 male Wistar rats consisted of Control (not exposed to cigarette smoke and given distilled water), KN (cigarette smoke and given distilled water), P1 (cigarette smoke and Calina Papaya Leaves Ethanolic extracts (CPLE) 100 mg/kg BW), P2 (cigarette smoke and CPLE 200 mg/kg BW), and P3 (cigarette smoke and given CPLE 300 mg/kg BW). Exposure to cigarette smoke and administration of extracts was carried out for 21 days. On day 22, the rats were euthanized and dissected to remove the lungs and trachea. Organs were prepared using the paraffin method and hematoxylin-eosin staining. Parameters consisted of histopathological observations of the lungs and trachea. All data parameters were analyzed using a one-way ANOVA test and Duncan's advanced test ($p<0.05$). The results showed that there was an improvement in histopathology of the lungs and trachea at a dose of 200 mg/Kg BW compared to other doses ($p<0.05$). The conclusion shows that the Calina papaya leaves ethanolic extracts have the potential as a source of antioxidants in improving the respiratory organs of Wistar rats after being exposed to cigarette smoke.*

Keywords : Antioxidants; Calina Papaya Leaves; Ciggarette Smoke; Lungs; Trachea

INTRODUCTION

Indonesia is one of the countries with high cases of respiratory health problems, one of which comes from smoke pollution. This country has several large cities with considerable exposure to air pollution, including vehicle fumes, factory fumes, and cigarette smoke. Cigarette smoke is a problem that needs to be taken seriously because the number of smokers in the last five years has increased. The data shows that the population aged over 15 years, which was originally 7.2% rose to 9.1%. (Kemenkes, 2018). Indonesia has even become the highest contributor to deaths due to cigarette consumption, which is around 225,700 deaths every year (WHO, 2019).

Cigarettes contain several dangerous chemical compounds such as CO, tar, nicotine, cyanide, ammonia, and dozens of other toxic compounds (Kemenkes, 2018). Some of these chemical compounds arise from the combustion process and become free radicals (Angelis et al., 2014). Free radicals cause health problems such as asthma, Chronic Obstructive Pulmonary Disease (COPD), cancer of the

respiratory tract, and stroke if they are in the body in the long term (Kemenkes, 2018). COPD is a complex disease involving both environmental and genetic factors. Cigarette smoke is one of the environmental factors that can cause COPD (15-20%) (Gershon et al., 2015). The prevalence of COPD in Indonesia according to Riskesdas in 2015 was 3.7% (4.2% male and 3.3% female), so that cigarette smoke can be a major risk factor for COPD (~~,~~ Suryadinata, 2018).

At the cellular level, cigarette smoke can increase the number of goblet cells, increase mucus production, and can damage cilia in the respiratory epithelium (Suryadinata et al., 2016; Kristiawan et al., 2017). High mucus production causes narrowing and obstruction of the airways and triggers ciliary abrasion so that the mechanism of cleaning the respiratory tract from foreign particles does not work optimally. Cigarette smoke also causes damage to the lungs in the form of inflammatory cell infiltration, pulmonary edema, and destruction of the alveolar septum (Silva & Bercik, 2012).

High levels of free radicals in the body due to cigarette smoke can be removed by antioxidant activity. Antioxidants are compounds that can inhibit the occurrence of oxidation reactions (Membri et al., 2021). Antioxidant compounds are found in many parts of herbaceous plants, such as leaves, fruit, and stems. One of the herbal plants that contain various kinds of antioxidant compounds is papaya (*Carica papaya* L.) (Setiawan et al., 2021[a]). Papaya leaves contain several antioxidant compounds such as alkaloids, triterpenoids, steroids, flavonoids, saponins, and tannins (Iskandar et al., 2020; Setiawan et al., 2021[b]). Papaya leaves also contain vitamin C as much as 16.29 mg/100g, these compounds can provide a protective effect against goblet cell hyperplasia in the respiratory tract (Suryadinata et al., 2016; Purlinda et al., 2020).

One of the papaya varieties cultivated in Indonesia is the Calina variety (IPB 9/California). This variety is the result of the papaya plant breeding Center for Tropical Fruit Studies (PKBT)-IPB (Setiawan et al., 2021[c]). These plants are cultivated for their fruit, while other plant parts such as leaves, stems, and roots are rarely used. There have not been many studies and scientific information regarding the potential of Calina papaya leaves as a source of natural antioxidants. Therefore, this study aimed to determine the effect of the Calina papaya leaf extract on the histopathology of the respiratory tract of wistar rats after exposure to cigarette smoke.

MATERIALS AND METHODS

The research was conducted at the Laboratory of Animal Structure and Physiology, Faculty of Applied Science and Technology, Universitas Ahmad Dahlan in January-Juni 2022. Calina papaya leaves were taken from the Vegetable Plantation of the hamlet of Druwo, Bangunharjo, Sewon, Bantul, DIY. The research has been approved by the UAD Ethics committee number 012101004. The determination of the Calina papaya plant was carried out at the Plant Systematics Laboratory, Faculty

of Biology, Universitas Gadjah Mada (No: 014900/S.Tb/x/2020) with the result that the scientific name of the papaya was *Carica papaya* L.

Calina Papaya Leaf Ethanol Extract

Calina papaya leaves (7 kg, old leaves, and dark green color) were washed with water, then dried in the hot sun, and covered with a black cloth for 5 days. The dried leaves were then blended until smooth, then sieved using a sieve number 100, so that 626 g of simplicia powder was obtained. The simplicia was then macerated using 96% ethanol for 3 days, and filtered using Whatman paper. The filtrate was then evaporated using a rotary vacuum evaporator (temperature 60°C and speed 85 rpm) until a semi-viscous extract was obtained. The extract was then placed in a water bath with a temperature of 70°C until a thick extract was formed. The Calina papaya leaf extract was then tested for chemical compounds using the phytochemical test to determine the phytochemical compounds (Table 1).

Animal Treatment

The study used 25 Wistar rats which were divided into 5 treatments with 5 replications. Male rats (±12 weeks old, weight ±185 g) were acclimatized for 1 week and kept intensively in cages measuring 50 cm x 40 cm x 15 cm, temperature ± 26 °C and humidity 70%. Rats were given BR 2 feed and drinking water ad libitum. Body weight measurements were carried out at the age of 0, 7, 14, and 21 days using a digital scale to obtain the average body weight used in calculating the dose of extract for each treatment.

The treatment of exposure to cigarette smoke used kretek cigarettes (34-65 mg tar, 1.9-2.6 mg nikotin, and 18-28 mg CO) as much as 3 sticks/day for each treatment for 21 days with a smoking chamber. The administration of cigarette smoke consisted of a negative control group (KN), Treatment 1 (P1), Treatment 2 (P2), and Treatment 3 (P3), while the control group was not exposed to cigarette smoke. After giving cigarette smoke, they were given a pause of 10 minutes so that the rats could inhale the rest of the cigarette smoke and then return them to their respective cages.

After 2 hours of exposure to cigarette smoke, the rats were then given Calina papaya leaves extracts dissolved in 1 ml of distilled water and given oral gavage for 21 days. The extract consisted of P1 with a dose of 100 mg/kg BW/day, P2 with a dose of 200 mg/kg BW/day, and treatment 3 (P3) with a dose of 300 mg/kg BW/day, while the Control and KN were given 1 ml of distilled water without extract.

Tissue preparations and histopathological observations of respiratory organs

On day 22, mice were anesthetized using 10% ether and sacrificed with neck dislocation for lung and trachea collection. The lungs and trachea were taken using a dissecting set, then the lungs were weighed using a digital scale to determine the organ weights and ratios. The trachea and lungs were then washed using 0.9% NaCl and fixed using 10% BNF to make tissue preparations using the paraffin

method (HE staining). The tissue preparations were then observed using an Olympus CX23 microscope and Optilab with a magnification of 10x and 40x. Parameters observed in the lungs consisted of the area of inflammatory cell infiltration, the number of cells with edema, and the diameter of the alveoli. Tracheal observation parameters consisted of tracheal lumen diameter, the height of tracheal surface epithelial cells, and the number of goblet cells. All parameters were measured using the Image Raster application.

Data analysis

All parameters data were analyzed using one-way ANOVA and continued using the Duncan test to determine differences between groups ($p< 0.05$).

RESULTS AND DISCUSSION

The results showed that Calina papaya leaves extracts to have several phytochemical compounds that can repair tissue damage after exposure to cigarette smoke. A phytochemical compound test showed that the Calina papaya leaves ethanolic extracts to contain several phytochemical compounds such as alkaloids, flavonoids, tannins, steroids, and saponins (Table 1). Secondary metabolite compounds in the Calina papaya leaves ethanolic extracts can act as antioxidants in protecting cells from the effects of free radicals caused by cigarette smoke (Nugroho & Sari, 2018). The effect of free radicals on cigarette smoke can reduce the body weight of rats in the treatment, it can be seen in the KN group which has a low weight compared to other treatments (Table 2). Antioxidant activity can counteract free radicals, thereby increasing the body weight of rats in the treatment given Calina papaya leaf extracts. The saponin compounds in the extracts increase the permeability of cell walls in the intestine so that the absorption of nutrients in food is more optimal (Irwani & Candra, 2016). This can be seen from the body weights of rats at P2 and P3 which were higher than those exposed to cigarette smoke (KN) ($p< 0.05$) (Table 2).

Table 1. Phytochemical compounds of Calina papaya leaf extract

Identification	Composition	Detection	Stain Color	Results
Alkaloids	Chloroform: Methanol: Ammonia (90:10:1)	Dragendrof	Orange	+
Flavonoids	Butanol: Ethyl acetate: Formic acid: Water (5:5:2:1)	Sitroborat	Yellow	+
Steroids	Hexan: Ethyl acetate (5:1)	Anisaldehid	Red	+
Saponins	Chloroform: Methanol: Water (100:13.5:10)	Lieberman Burchard	Green	+
Tannins	Ethyl acetate: Methanol: Water (100:13.5:10)	$FeCl_3$	Black	+

Notes: (-) does not contain compounds, (+) contains compounds.

Rats in the cigarette smoke exposure (KN) treatment showed lower body weight and lung ratio compared to other treatments ($P<0.05$). Cigarette smoke contains about 4000 chemical compounds that

are harmful to the body such as CO, tar, nicotine, cyanide, ammonia, and dozens of other toxic compounds (Kemenkes, 2018). Nicotine in cigarette smoke can reduce appetite and reduce body weight (Rahma et al., 2019; Huriyati & Amareta, 2020). Body weights and lung ratios in Controls, P2, and P3 (Table 3) were higher than those in KN, this indicates that the Calina Papaya Leaves extracts can inhibit the decrease in body weight and organ ratios due to exposure to cigarette smoke.

Treatment	Body Weight (g)	Lung Weight (g)	Lung Ratio (%)
K	196.50 ± 22.25^{b}	1.21 ± 0.16^{a}	0.0058 ± 0.00^{a}
KN	154.25 ± 9.91^{a}	1.11 ± 0.07^{a}	0.0067 ± 0.00^{b}
P1	170.75 ± 8.10^{a}	1.11 ± 0.06^{a}	0.0060 ± 0.00^{a}
P2	205.25 ± 15.20^{b}	1.26 ± 0.12^{a}	0.0056 ± 0.00^{a}
P3	201.50 ± 12.78^{b}	1.24 ± 0.10^{a}	0.0057 ± 0.00^{a}

Note: Different superscripts within the same column indicate significant differences ($p<0.05$). K: Control (100mg/kg BW extract and not exposed to cigarette smoke), Negative Control/KN (exposure to cigarette smoke), P1 (100mg/kg BW extract and exposure to cigarette smoke), P2 (200mg/kg BW extract and exposure to cigarette smoke), P3 (300mg/kg BW extract and exposure to cigarette smoke).

The results of the study in the Negative Control Group (KN) showed an increase in the area of inflammatory cell infiltration, the amount of pulmonary edema, and a significant alveolar diameter when compared to those given the extracts (Table 3). Exposure to cigarette smoke triggers oxidative stress, causing inflammation that causes inflammatory cell infiltration. Inflammation is indicated by the number of inflammatory cells that collect in the alveoli of the lungs (Figure 1). Oxidative stress from cigarette smoke exposure activates alveolar macrophages and neutrophils. This can trigger the inactivation of 1-anti-trypsin (anti-protease) which causes proteases to increase and neutrophil activation. The excessive increase in neutrophils causes the degradation of parenchymal connective tissue in the lungs (causing edema). Edema is seen with the accumulation of fluid in the alveolus area (Figure 1) due to exposure to cigarette smoke. Degradation of alveolar cells due to oxidative stress also destroys the alveolar septum (Ardy et al., 2020; Nurliani et al., 2012; Tohomi et al., 2014).

Treatment	Inflammation area (μm^2)	Number of Pulmonary Edema cell	Alveolar Diameter (μm^2)
K	542.95 ± 1.96^{b}	3.75 ± 0.005^{a}	43.26 ± 0.60^{a}
KN	550.45 ± 4.54^{c}	5.25 ± 1.50^{b}	70.11 ± 3.84^{c}
P1	544.94 ± 1.36^{b}	4.75 ± 0.58^{ab}	68.81 ± 1.30^{c}
P2	524.71 ± 0.98^{a}	3.50 ± 0.58^{a}	40.94 ± 2.22^{a}
P3	543.99 ± 4.54^{b}	4.25 ± 0.50^{ab}	58.81 ± 0.45^{b}

Note: Different superscripts within the same column indicate significant differences ($p<0.05$). K: Control (100mg/kg BW extract and not exposed to cigarette smoke), Negative Control/KN (exposure to cigarette smoke), P1 (100mg/kg BW extract and exposure to cigarette smoke), P2 (200mg/kg BW extract and exposure to cigarette smoke), P3 (300mg/kg BW extract and exposure to cigarette smoke).

The results showed that P2 and P3 had the lowest area of inflammation, and the least amount of cell edema, with normal alveolar diameter, compared to KN ($p<0.05$). The results also showed that the

area of inflammation and the number of edema cells in P2 was better than the Control (K). Phytochemical compounds in Calina papaya leaves extracts such as flavonoids, steroids, saponins, and tannins can reduce damage to lung tissue after exposure to cigarette smoke (Abdelmohsen et al., 2012). Flavonoids are thought to inhibit the release of inflammatory cells in the form of alveolar macrophages and neutrophils (Hu et al., 2013). Saponins can act as immunostimulators that act as protease inhibitors for the restriction of released cytokines (TNF-α, IL-1β, IL-2, and -γ) (Idrus, 2014). Steroids can maintain the balance of cell membranes from ROS (Hardiningtyas et al., 2014).

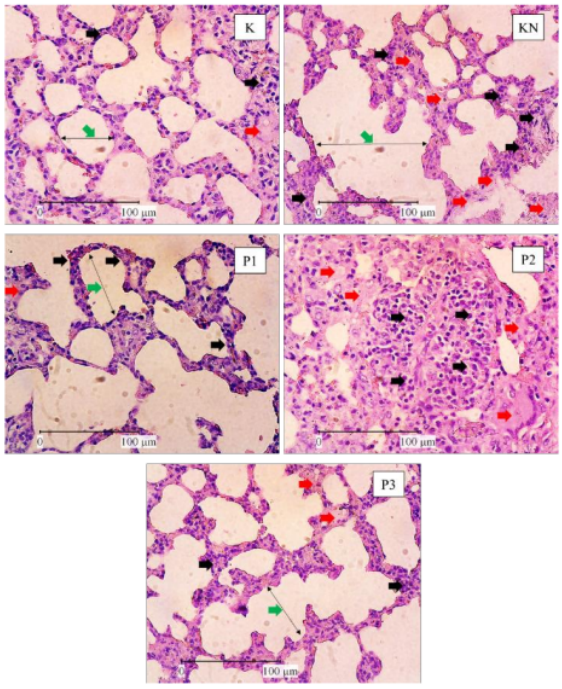

Figure 1. Histopathological structure of the lungs in the treatments. Note: K/Control (0 mg/kg BW extract and not exposed to cigarette smoke), KN/Negative Control/KN (exposure to cigarette smoke), P1 (100mg/kg BW extract and exposure to cigarette smoke), P2 (200mg/kg BW extract and exposure to cigarette smoke), P3 (300mg/kg BW extract and exposure to cigarette smoke). Black arrow (area inflammation), red arrow (cell edema), green arrow (alveolar diameter). Haematoxylin-Eosin staining. Scale bar 100 μm.

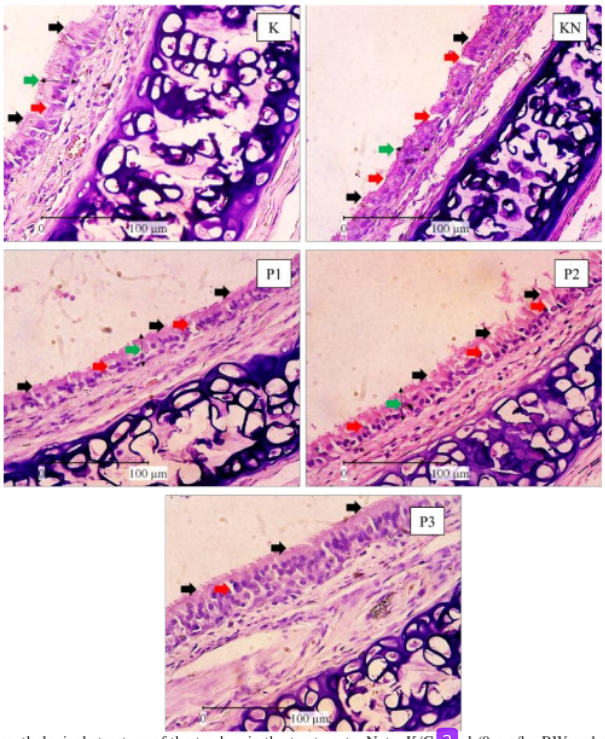

Figure 2. Histopathological structure of the trachea in the treatments. Note: K/Control (0 mg/kg BW and not exposed to cigarette smoke), Negative Control/KN (exposure to cigarette smoke), P1 (100mg/kg BW extract and exposure to cigarette smoke), P2 (200mg/kg BW extract and exposure to cigarette smoke), P3 (300mg/kg BW extract and exposure to cigarette smoke). Black arrows (ciliary cells), red arrows (goblet cells), and green arrows (epithelial cell height). Haematoxylin-Eosin staining. Scale bar 100 μm.

Table 4. Histopathological structure of the trachea in the treatment

Treatment	Tracheal lumen diameter (μm)	Epithelial cell height (μm)	Number of Goblet Cells
K	3061.54 ± 122.93^{c}	29.26 ± 12.70^{c}	58.75 ± 9.11^{a}
KN	2271.27 ± 137.21^{a}	11.72 ± 3.78^{a}	95.75 ± 20.02^{b}
P1	2363.15 ± 142.94^{a}	16.68 ± 7.28^{ab}	83.25 ± 22.23^{ab}
P2	2721.53 ± 219.13^{b}	19.06 ± 6.69^{ab}	73.50 ± 9.54^{ab}
P3	3007.04 ± 292.09^{bc}	26.11 ± 6.56^{c}	58.25 ± 16.15^{a}

Note: Different superscripts within the same column indicate significant differences ($p<0.05$). K: Control (0 mg/kg BW and not exposed to cigarette smoke), Negative Control/KN (exposure to cigarette smoke), P1 (100mg/kg BW and exposure to cigarette smoke), P2 (200mg/kg BW and exposure to cigarette smoke), P3 (300mg/kg BW and exposure to cigarette smoke).

Exposure to cigarette smoke also causes damage to the tracheal organs of mice. The KN group showed a decrease in lumen diameter and epithelial height, and an increase in the number of goblet cells

(p<0.05) (Table 4). Exposure to cigarette smoke triggers inflammation by stimulating the formation of proinflammatory cytokines such as IL-6, IL-2, and Tumor Necrosis Factor Alpha (TNF-α). This stimulates an increase in the number of goblet cells in the surface layer of the trachea and results in a narrowing of the diameter of the lumen of the trachea (Russi et al., 2013; Angelis et al., 2014; Suryadinata, 2018). Figure 2 shows that exposure to cigarette smoke reduces the number of epithelial cilia so that it can reduce its function in protecting the surface of the tracheal mucosa (Kristiawan et al., 2017). Damage to cilia can cause structural changes and induce epithelial cell apoptosis, thereby affecting the decrease in epithelial height on the surface of the trachea (Wira et al., 2018).

The administration of papaya leaf ethanol extract gave a significant improvement in epithelial height at P1, P2, and P3 (p< 0.05) (Table 4). The results showed that P3 showed the highest results in tracheal diameter, epithelial cell height, and a decrease in the number of goblet cells compared to other treatments (p<0.05). Phytochemical compounds in papaya leaves can protect cells from free radical attack by donating one of their electrons to compounds that are oxidant so that the activity of these oxidant compounds can be inhibited, thereby reducing the degradation of the surface layer of the trachea (Kristiawan et al., 2017). The decrease in the number of goblet cells at P3 showed that the ethanol extract of papaya leaves at a dose of 300 mg/Kg BW showed an improvement in the tissue structure of the epithelial layer of rats exposed to cigarette smoke. Flavonoid compounds and tannins in the ethanol extract of Calina papaya leaves are known to prevent oxidative stress. These compounds can ward off free radicals and inhibit the increase in levels of ROS by donating electrons to free radicals into a more stable form (Setiawan et al., 2020; Yatalaththov et al., 2021). Exposure to cigarette smoke that is given repeatedly is thought to cause an increase in the mitotic index in the respiratory tract epithelium of experimental animals and then it will cause hyperplasia of goblet cells resulting in an increase in mucus secretion in goblet cells (Angelis et al., 2014). The ethanol extract of Calina papaya leaves at a dose of 300mg/Kg BW was able to reduce the number of goblet cells compared to other treatments (p<0.05). The content of flavonoid compounds in the extract also plays an important role in inhibiting the formation of proinflammatory cytokines (IL-12 and TNF- expression), preventing cell damage and inflammation (Rousdy et al., 2022). Alkaloid compounds can also stop lipid peroxidation which will protect cells from free radicals (Pratiwi et al., 2021).

CONCLUSION

The results of this study concluded that the administration of Calina papaya leaves ethanolic extracts at doses 200 mg/kg BW and 300mg/kg BW for 21 days was able to repair damage to the respiratory organs consisting of the lungs and trachea of wistar rats due to exposure to cigarette smoke.

AUTHOR CONTRIBUTION

H.S and S.W.W. supervised the process and wrote the manuscript. H.S, A.S.A.H, and E.V.M carried out the experiment and collected and analyzed the data. I.L.I.P proofread and edited the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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